Accepted Manuscript

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PII: S0958-6946(17)30027-4

DOI: 10.1016/j.idairyj.2016.12.016

Reference: INDA 4139

To appear in: *International Dairy Journal*

Received Date: 23 September 2016

Revised Date: 21 December 2016

Accepted Date: 21 December 2016

Please cite this article as: Barry, K.M., Dinan, T.G., Kelly, P.M., Selective enrichment of dairy phospholipids in a buttermilk substrate through investigation of enzymatic hydrolysis of milk proteins in conjunction with ultrafiltration, *International Dairy Journal* (2017), doi: 10.1016/j.idairyj.2016.12.016.

Selective enrichment of dairy phospholipids in a buttermilk substrate through investigation of enzymatic hydrolysis of milk proteins in conjunction with ultrafiltration

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ABSTRACT

Extensive enzymatic hydrolysis of milk proteins in reconstituted buttermilk powder was combined with ultrafiltration to generate a phospholipid (PL) enriched fraction with maximum permeation of hydrolysed peptides. Buttermilk, naturally high in PLs, is the ideal substrate for enrichment of these bio- and techno- functionally active compounds. A 7.8 fold increase in PL was achieved in the 50 kDa retentate; 6.16 ± 0.02% total PL compared with 0.79 ± 0.01% in the starting substrate, an increase considerably greater than previously reported. Total lipid content (% dry matter) increased 6.3 fold in the retentate, 43.43 ± 0.61%, from the starting substrate, 6.84 ± 0.17%. This combined strategic approach enabled maximum enrichment of PLs with no transmission of lipid material into the permeate, 0.09 ± 0.02% total lipid, and non-detectable levels of PLs recovered in the permeate, 0.00 ± 0.01% total PL.

1. Introduction

Cream churning during the butter manufacture process disrupts the natural emulsifying layer of the milk fat globule and releases fragments of the milk fat globule membrane (MFGM), namely polar lipids and membrane proteins, into the aqueous (serum) phase that characterises the by-product buttermilk (BM) (Corredig, Roesch & Dalgeleish, 2003; Morin, Jiménez-Flores, & Pouliot, 2007). While similar to skim milk in terms of protein (casein and whey), lactose and minerals (Corredig & Dalgleish, 1997; Keenan, & Dylewski, 1995; Vanderghem et al., 2010), BM is distinguishable by its marginally higher fat content, 2.80% fat compared with 0.74% in skim milk (Barry, Dinan, Murray & Kelly, 2016), arising from destabilisation of the milk fat globules and the release of MFGM-based polar lipids (Britten, Lamothe, & Robitalle, 2006; Sodini, Morin, Olabi, & Jiménez- Flores, 2006). The concentration of these polar lipids, phospholipids (PLs), in BM has been reported to be up to 15 times greater than that of whole milk (Barry et al., 2016; Christie, Noble, & Davies, 1987; Rombaut, van Camp, & Dewettinck, 2005). Phospholipids constitute two groups of polar lipids, glycerophospholipids and sphingolipids (Avalli & Contarini, 2005; Contarini & Povolo, 2013; Rombaut et al., 2005). However, not all sphingolipids constitute glycerophospholipids, e.g., sphingosine, due to the absence of the phosphate group. The major glycerophospholipids are phosphatidyethanolamine (PE) and phosphatidylcholine (PC), while phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA) are present in relatively minor amounts (Barry et al., 2016). Sphinomyelin (SM) represents the major sphingolipid (Barry et al., 2016; Contarini & Povolo, 2013; Rombaut, Dewettinck, & van Camp, 2007a). Hence, there is a growing interest in BM as an ideal substrate for the development of enriched PL concentrates.

Quantitatively, BM production is estimated to be similar to that of butter, which was reported to be approximately 5.2 million tonnes worldwide in 2013 (FAOSTAT, 2015). Previously considered a low value by-product from butter manufacture, BM is now widely used in the food and beverage industry due to the inherent amphiphilic nature of its PLs (with their hydrophobic tail and hydrophilic head group: Contarini & Povolo, 2013; Dwetteinck et al., 2008) that is mainly responsible for the functional and technological properties of BM, such as emulsification and stabilisation (Corredig &

Dalgleish, 1997; Ferreiro, Martinez, Gayoso, & Rodriguez-Otero, 2016; Kuchta, Kelly, Stanton, & Devery, 2012).

In addition, there are numerous biological and nutritional properties associated with PLs, particularly those derived from dairy sources due to their higher content of PS and SM (Barry et al., 2016; Burling & Graverholt, 2008). SM and its metabolites have been shown to exhibit anticarcinogenic activity, in particular suppressing colon carcinogenesis (Berra, Colombo, Scottocornola, & Giacosa, 2002; Hertervig, Nilsson, Cheng, & Duan, 2003, 2005; Kuchta- Noctor, Murray, Stanton, Devery, & Kelly, 2016; Parodi, 2003; Spitsberg, 2005). PLs have also been associated with cognitive performance and improvement, in particular the biological activity associated with PS has been utilised in the treatment of Alzheimer's disease (Burling & Graverholt, 2008; Pepeu, Pepeu, & Amanducci, 1996). Other positive health benefits associated with PLs are linked to reduced incidence of cardiovascular disease, suppression of multiple sclerosis, myelination of the central nervous system, depression and stress (Contarini & Povolo, 2013; Dewettinck et al., 2008; Oshida et al., 2003). Supplementation of infant formulas with PLs has been shown in recent years to have a positive effect on both the neurodevelopment and immunological defences of the infant (Timby, Domellöf, Hernell, Lönnerdal, & Domellöf, 2014; Timby et al., 2015).

Due to its widespread availability, low cost and functionality, BM is an appropriate starting substrate to consider for production of a PL concentrate. Membrane separation processes have been explored by various groups in the course of enriching the concentration of valuable MFGM material in BM. Microfiltration, because of its selectivity to remove caseins, has been extensively researched (Holzmüller & Kulozik, 2016); however, the transmission of MFGM components has been shown to be dependent on a number of factors such as pore size, processing temperature, pH, type of BM and also the membrane filtration configuration (Astaire, Ward, German, & Jiménez- Flores, 2003; Dewettinck et al., 2008; Morin, Jiménez-Flores, & Pouliot, 2004; Morin, Pouliot, & Jiménez-Flores, 2006; Rombaut, Dejonckheere, & Dewettinck, 2007a; Surel & Famelart, 1995). The similarity in size of casein micelles and PLs has been reported to be a major obstacle during selective isolation of MFGM components by membrane filtration. Apparent interactions between casein micelles and MFGM are thought to be partially responsible for the poor separation of PLs by microfiltration

(Morin et al., 2007). This has resulted in complementary measures such as cream washing in conjunction with ultrafiltration (UF) being explored to facilitate maximum removal of the skim milk proteins, lactose and minerals (Morin, Britten, Jiménez-Flores & Pouliot, 2007). Other measures include the investigation of pre-disruption, coagulation and precipitation of the casein fraction prior to filtration (Corredig et al., 2003; Roesch, Rincon, & Corredig, 2004; Rombaut et al., 2007a; Sachdeva & Buchheim, 1997). Sachdeva and Buchheim (1997) induced rennet coagulation of the caseins prior to MF, but suffered a 20% loss of the PL in the rennet curd (Sachdeva & Buchheim, 1997). Corredig et al. (2003), Roesch et al. (2004) and Rombaut et al. (2007a) improved the MF transmission of casein as a result of disruption by added citrate, but experienced losses of MFGM material due to membrane fouling and pore blocking (Rombaut, Dejonckheere, & Dewettinck, 2006). Another avenue of investigation was cream washing with skim milk ultrafiltrate prior to butter/buttermilk production (Britten et al., 2007; Holzmüller, Müller, Himbert, & Kulozik, 2016; Morin et al., 2007b). In this instance, washing cream prior to churning reduced BM protein content by 74–80%, but at the expense of severe PL losses (Britten et al., 2008). The type of BM used also influences the transmission of MFGM components. A 50% decrease in transmission of PLs has been reported in fresh pasteurised BM compared with reconstituted BM which is attributed to differences in processing (Morin et al., 2004). Whey BM, a by-product of cheese production, is a desirable substrate for MFGM isolation due to the absence of casein micelles, but of less interest commercially due to the low production volumes of whey cream and also the susceptibility of whey butter to lipid oxidation (Morin et al., 2007a).

This study set out to develop a process to maximise both enrichment and recovery of PL material using reconstituted buttermilk powder as a standard substrate for all experimental studies, due to its availability, utilising a combination of enzymatic hydrolysis and UF fractionation. UF has not been as widely investigated as MF for PL enrichment, but may serve as an avenue to overcome the unsatisfactory results obtained by fractionation by means of microfiltration (Morin et al., 2007a). In particular, the study pushes the boundaries of previous work done with enzymatic hydrolysis of buttermilk prior to microfiltration. In this case, more extensive protein hydrolysis is aimed for by initially screening commercial proteases on the basis of their capacity to generate smaller peptides

capable of permeating UF membranes, thereby reducing the protein content of the retentate and higher levels of polar lipid enrichment. UF, with its smaller pore size, should ensure minimal transmission of the PLs compared with transmission experienced during MF (> 45%; Morin et al., 2004) and thus maximise recovery of these complex bio- and techno- functional compounds in the retentate.

2. Materials and methods

2.1. Materials

Spray-dried buttermilk powder was sourced from Tipperary Co-operative (Tipperary, Ireland). Compositional analysis of the powder by standard IDF protocols determined 6.84% fat, 31.40% protein, 48.00% lactose and 7.40% minerals. The digestive enzymes Alcalase® (EC3.4.21.62) and Neutrase® (EC3.4.22) were procured from Novozymes (Bagsværd, Denmark). Alcalase has a minimum activity of 2.4 Anson units (AU) per gram of composition (2.4 AU g^{-1}) at pH 7.5, with the optimum conditions for Alcalase activity within 35–60 °C and pH 7–9. Neutrase is a metalloprotease with a minimum activity of 0.8 AU g^{-1} protease at pH 7 with optimal conditions for neutrase activity between 40–50 °C and pH 5–7. The digestive enzyme complex Corolase PP® (CPP) (EC 3.4.21.4), was procured from AB Enzymes GmbH (Darmstadt, Germany). CPP has a minimum activity of 2500 units with haemoglobin as a substrate (UHb) and 220,000 Lohlein-Volhard units (LVU) g^{-1} at pH 8.0.

Vivaflow^{TM} 200 polyethersulfone (PES) cross-flow ultrafiltration cassette membranes (molecular mass cutoffs of 50 kDa and 100 kDa) were purchased from Sartorius Stedim (Sartorius Stedim Ireland Ltd., Dublin, Ireland). Acetonitrile was procured from ThermoFisher Scientific (Waltham, MA, USA). All other chemicals were HPLC grade, > 99.9 % and were purchased from Sigma Aldrich (Arklow, Ireland)

2.2. Enzymatic hydrolysis of buttermilk powder protein fraction

The degree of hydrolysis (DH) is defined as the number of peptide bonds (*h*) cleaved as a percentage of the total peptide bonds (*h_{tot})* and is related to base consumption due to proton release during the hydrolysis reaction according to Eq. 1 (Adler-Nissen, 1986):

$$\%DH = 100\ x\ B\ x\ N_B\ x\ \frac{1}{\alpha}\ x\ \frac{1}{MP}\ x\ \frac{1}{h_{tot}} \quad (1)$$

where B is the volume of base in mL, N_B is the normality of the base, 1/α is the average degree of dissociation of the α-NH groups (= 1.13 at pH 8.0, 50 °C), MP is the molecular mass of the protein and h_{tot} is the number of peptide bonds in the protein substrate (= 8.2 for casein/whey mix).

Investigative screening of a number of proteases, either individually or in combination, was carried out to determine the most proteolytic enzyme in terms of greater amount of smaller molecular mass peptides generated. These included CPP, Neutrase and Alcalase as well as Alcalase in combination with CPP or Neutrase. For analysis of the changes in molecular mass distribution and peptide formation during the hydrolysis reaction for each individual enzyme or enzyme combination, aliquots of the reaction were subtracted at different DH points (determined using Eq. 1) and the enzyme activity was terminated by dilution in 0.1% (v/v) trifluoroacetic acid (TFA). The hydrolysates were then analysed via size exclusion chromatography to determine the enzyme that generated the greatest degree of smaller molecular mass material. Going forward, all hydrolysis experiments described will refer to the use of Alcalase as the proteolytic enzyme unless otherwise stated.

For all hydrolysis reactions, buttermilk powder (BMP) was reconstituted to 10% total solids in deionised water, 800 mL, and allowed to hydrate overnight at 4 °C with gentle agitation (300 rpm). The BMP dispersions, with an original pH of 6.45, were adjusted to pH 8.0 with 4 M NaOH and heated to 50 °C in a water bath. Enzymatic hydrolysis was initiated by addition of Alcalase dissolved in dH_2O, and the pH was controlled throughout by titration with 0.2 M NaOH using a Metrohm 842 Titrando pH stat dosing unit (Metrohm Ltd., Herisau, Switzerland). The reaction was agitated throughout utilising an over-head stirrer at 300 rpm. DH ranging from 1% to 25% was investigated, with percentage DH (%DH) selection based on the optimum digestion of the milk proteins. Termination of the reaction by enzyme inactivation achieved through heating the hydrolysate to 80 °C for 20 min or by pH inactivation with 0.1% (v/v) TFA was investigated to determine the effects of the

enzyme inactivation process on the final hydrolysate. All hydrolysate reactions were performed in triplicate.

Determination of the end-point DH was measured by a modification of the method described by Adler-Nissen (1979) for the trinitrobenzenesulfonic acid (TNBS) reaction. TNBS involves measurement of the reaction of liberated α-amino groups (α-NH_2) with 2,4,6-trinitrobenzene-1-sulfonic acid. Briefly, a standard curve was prepared using L-leucine at concentrations ranging from 20 to 250 mg L^{-1}. Samples with a 3 mg mL^{-1} protein concentration were prepared in 1% SDS to which 0.2125 M phosphate buffer was added. 1% SDS alone was used as a blank. To both the test samples and blank, 2 mL of TNBS was added, with the exclusion of light, and the reaction was incubated at 50 °C for 1 h. Termination of the reaction was achieved through addition of 4 mL 0.1 M HCL, and the samples were cooled for 30 min following which the absorbance at 340 nm was read using a Varian Cary 1 dual beam UV- visible spectrometer (Varian Ltd., Walton-on-Thames, UK).

DH values were determined using the following equation (Spadaro, Draghetta, del Lama, Camargo, & Greene, 1979) (Eq. 2)

$$\%DH = \frac{AN_2 - AN_1}{N_{pb}}\ x\ 100 \qquad (2)$$

where, AN_1 is the amino nitrogen content of the unhydrolysed protein substrate (mg g^{-1} protein), AN_2 is the amino nitrogen content of the hydrolysed protein substrate and N_{pb} is the nitrogen content of the peptide bonds in the protein substrate (mg g^{-1} protein). DH values were compared with the pH stat determined values.

To determine optimum enzyme to substrate ratio (E:S) for the hydrolysis, reactions were performed at E:S ratios ranging from 0.1% to 2%, with reaction times dependent on DH and E:S, calculated using Eq. 1, under the hydrolysis conditions previously described.

2.3. Ultrafiltration of BMP hydrolysates

Hydrolysates were subjected to membrane fractionation using Vivaflow™ 200 PES cross-flow cassette UF membranes (Sartorius Stedim); molecular mass cut-offs of 100 kDa and 50 kDa

were investigated to determine optimum fractionation of the hydrolysate. UF was carried out at 50 °C with a membrane inlet pressure of 1 Bar maintained throughout the process. Two diafiltration steps with 400 mL dH_2O (half the volume of the starting BMP hydrolysate) were performed to achieve a volume concentration factor of 8. Retentates and permeates were concentrated using a Buchi rotary evaporator (Büchi, Flawil, Switzerland) and subsequently freeze-dried using a FreeZone® Plus 12 L (Labconco, Kansas City, MO, USA). UF of BMP hydrolysate was carried out in triplicate.

2.4. Chromatographic analysis of BMP hydrolysates and UF fractions

For molecular mass characterisation and observation of the loss of native protein, HPLC was carried out using a Waters 2695 separation module and a Waters 2487 dual wavelength absorbance detector with Waters Empower software (Milford, MA, USA). Column eluates for protein analysis were monitored at 214 nm.

Size exclusion chromatography (SEC) was carried out according to the method of O'Loughlin et al. (2013). Briefly, 20 µL of sample with 2.5 mg mL^{-1} protein concentration in Milli-Q water was injected onto a TSK Gel G2000SW, 7.8 mm × 600 mm column (Tosoh Bioscience GmbH, Stuttgart, Germany) with an isocratic gradient of 30% acetonitrile (MeCN) containing 0.1% TFA and a run time of 60 min. A molecular mass curve was generated using the following protein standards at 2.5 mg mL^{-1} protein (20 µL injection volume): α-lactalbumin. β-lactoglobulin A and B, bovine serum albumin (BSA), lactoferrin and CMP (from Sigma Aldrich) and ribonuclease A, cytochrome C, Aprotinin, Bacitracin, His–Pro–Arg–Tyr, Leu–Tyr–Met–Arg, Bradykinin, Leu–Phe and Tyr–Glu (Bachem AG, Bubendorf, Switzerland). All samples and standard were pre-filtered through glass filters and 0.22 µm low protein binding membrane filters (Sartorius Stedim) prior to HPLC analysis.

2.5. Lipid extraction, separation and quantification of the BMP phospholipids

Separation and quantification of PLs in the BMP hydrolysate and UF fraction involved extraction of crude lipid by Folch and subsequent analysis and quantification via HPLC according to Barry et al. (2016). Briefly the crude extract was dissolved in $CHCl_3$:MeOH (88/12, v/v) with 25 µL of the sample applied to the HPLC-detection system, Waters 2695 separation module (Waters) coupled to a corona charged aerosol detector (CAD) (Thermo Scientific, Hemel Hempstead, UK). PL analysis was carried out on a 3 µm particle diameter Prevail Silica column (Grace Sciences, Labquip, Dublin, Ireland) under linear gradient elution conditions with the ratio in volume of solvent A to B as follows; 96:4 at T = 0 min, 25:75 at T = 20 min, 6:94 at T = 21 min and 96:4 at T = 22 min and maintained until T = 27 min at a flow rate of 0.50 mL min^{-1}. PL recovery was determined by quantifying with respect to calibration curves generated for each individual PL species. Operating pressure of the CAD was maintained at 241.32 kPa with N_2 as the nebulising gas with a flow rate of 2.1 L min^{-1}. All lipid extractions were performed in triplicate and all samples were pre-filtered through 0.20 µm Hydrophobic Fluoropore™ (PTFE) membrane filters (Merek Millipore Ltd. Cork, Ireland) prior to HPLC analysis

2.5. Compositional analysis of BMP UF fractions

Compositional analysis was performed in duplicate on all samples using the standard IDF/ISO or AOAC methods for determination of % protein (ISO, 2014), % total solids/moisture and ash (ISO, 2010) and lactose (AOAC, 2012); % fat, performed in triplicate, was determined using the Folch method described by Barry et al. (2016).

2.6. Statistical analysis

Statistical analysis was performed with Minitab, version 15 (Minitab Ltd, Coventry, UK), with one-way analysis of variance (ANOVA) and Tukey Tests for both intra- and inter- sample variance.

3. Results and discussion

3.1. BMP hydrolysates and target degree of hydrolysis determination

In the current study, screening of a number of proteases, Neutrase®, Corolase PP®, and Alcalase®, both individually or in combination, was carried out to determine the most proteolytic enzyme with enzyme selection based on generation of the greatest degree of smaller molecular mass material. Each enzyme was investigated in terms of their ability to hydrolyse the BMP substrate, with the molecular mass distribution of each hydrolysate produced during the reactions analysed by SEC. From the chromatograms obtained (Fig. 1), Alcalase alone (Fig. 1a) proved to be the most proteolytic enzyme on the basis of smaller molecular mass material, of which it generated 89.84% <50 kDa in size (Table 1). The enzyme combinations, CPP + Alcalase and Neutrase + Alcalase, generated 68.26% and 73.17% < 50 kDa, respectively (Fig. 1d and 1e); however,there is a large portion of larger molecular mass material, > 50 kDa, 31.73% and 27.44% in the CPP + Alcalase and Neutrase + Alcalase hydrolysates, respectively. This may be due to residual unhydrolysed protein or aggregate formation with smaller molecular mass peptides through sulphide linkages (Mullally, O'Callaghan, Fitzgerald, Donelly, & Dalton, 1994). CPP alone (Fig. 1b) had the greatest amount of larger molecular mass material, 63.94% > 50 kDa (Table 1). The difference in the generation and amounts of smaller mass peptides indicates the different mechanisms of hydrolysis for each of the enzymes screened. Alcalase is an endoprotease of the serine type and acts as an esterase enabling it to catalyse stereoselective hydrolysis of amino esters and selective esters. Neutrase acts as a metalloprotease enabling the kinetic resolution of amino esters. CPP is a digestive complex also containing serine endoprotease activity and has been reported to have high chymotrypsin to trypsin activity ratio (Mullally et al., 1994). As a result of the screening, Alcalase (Fig. 1a) was selected as the enzyme going forward to produce the BMP hydrolysates that would be subjected to ultrafiltration.

As mentioned earlier (Section 2.2), the optimum %DH for the Alcalase digested hydrolysates was investigated with %DH selection based on the greatest digestion of the milk proteins. DH was

determined by the volume of base consumed as calculated using Eq. 1. Size exclusion chromatography (SEC) was utilised to characterise the change in molecular mass distribution of the hydrolysed protein over the course of the hydrolysis reaction and peptide formation at each DH point. From the resulting SEC chromatograms (Fig. 2), it was determined that a DH of 19% (Fig. 2c) ensured extensive hydrolysis of the milk proteins with almost complete digestion of the larger molecular mass material present in the BMP starting substrate (Fig. 2a), with a shift in mass from 92.10% >100 kDa in the BMP to 0.25% > 100 kDa in the BMP hydrolysate (Table 1). Extending the reaction to 25% DH had no significant change on the molecular mass distribution profile (Fig. 2d). Thus 19% DH was selected going forward as the end-point for the hydrolysis reactions as it ensured extensive hydrolysis of the proteinacious material such that the large body, 89.84% (Table 1), of smaller peptides (< 50 kDa) generated would facilitate peptide permeation during UF, and thus concentrate the lipid and in particular phospholipid fractions.

SEC was also utilised to investigate the effect that mode of enzyme inactivation, i.e., heat-induced (80 °C for 20 min) versus pH (0.1% TFA) had on the final hydrolysate. It was concluded (Fig. 3) that pH inactivation to pH 5 was the preferable method of enzyme inactivation, as aggregate formation (> 50 kDa) occurred following thermal treatment of the hydrolysate to 80 °C for 20 min (Fig. 3c), and this did not occur in the pH inactivated hydrolysate, Fig. 3b. Aggregate formation due to release of peptides during the hydrolysis reaction held together by non-covalent interactions has been comprehensively studied (Otte, Lomholt, Halkier, & Qvist, 2000). Altering the pH to 5 was sufficient to inactivate the enzyme. Alcalase is known to be quickly inactivated at pH 5 and above pH 11 (Douchet, Otter, Gauthier, & Foegeding, 2003).

3.2. Ultrafiltration of BMP hydrolysate

UF and diafiltration (DF) was carried out on the BMP hydrolysate to maximise permeation of the digested protein material, and thereby, reduce the amount of retained proteinaceous material in the phospholipid (PL)-containing retentate. Membrane cassettes with a choice of two cut-offs, i.e., 50 kDa and 100 kDa, were screened initially based on the profiles of their respective permeating

peptides. Each UF step was carried out in duplicate. From the SEC chromatograms obtained for both the 50 kDa and 100 kDa retentates (data not shown) it was determined that the molecular mass distribution profiles were not significantly different between the two UF membranes ($p < 0.01$, one-way ANOVA), and consequently, the 50 kDa membrane was selected to accomplish the selective concentration step in subsequent studies.

UF was carried out at 50 °C and inlet pressure of 1 bar. A 6-fold increase in lipid content of the retentate (50 kDa R), 43.43 ± 0.61% total lipid (dry matter, DM), compared with 6.84 ± 0.17% total lipid (DM) in the starting BMP material (Table 2). At the same time, a 2-fold reduction in total protein content of the BMP, from 31.40 ± 0.57% to 17.9 ± 0.19%, in the 50 kDa R was achieved as a result of increased UF protein permeation in the form of smaller peptide material (< 50 kDa) (Table 2). Molecular mass distribution profiling by SEC of the 50 kDa R and the 50 kDa permeate (50 kDa P) confirmed the deproteinising effectiveness of this combined hydrolysis/UF process (Fig. 4), e.g., a maximum absorbance of 0.108 mV for 50 kDa P (Fig. 4a) compared with 1.491 mV for 50 kDa R (Fig. 4b).

3.3. Phospholipid enrichment in a 50 kDa retentate following UF/DF of BMP hydrolysate

The degree of phospholipid (PL) enrichment achieved was determined through quantification of the PL profiles obtained from the BMP starting material, the 50 kDa R and the 50 kDa P according to the HPLC method outlined by Barry et al. (2016). Total lipid was extracted from each of the three substrates via the method of Folch, Lees, and Stanley (1957). A 6-fold increase in total lipid in the 50 kDa R with respect to the starting BMP was achieved by utilising the combined process of protein hydrolysis and UF/DF as already described (Section 3.2), 43.43 ± 0.61% total lipid and 6.84 ± 0.17% total lipid respectively (Table 2). This is significantly greater than that achieved previously. Roesch and Corredig (2002) obtained an ingredient with a total lipid increase from 8% to 16% in the final retentate following hydrolysis and UF. The extracted lipid was subsequently analysed by HPLC-CAD to determine the PL content. A PL concentration effect was observed between the BMP starting material and the 50 kDa retentate, 0.79 ± 0.00% total PL (summation of PI, PE, GluCer, PS, PC and

SM) and 6.16 ± 0.02% total PL, respectively, expressed as a percentage of the sample (Table 2). This demonstrates a 7.8 fold increase in the PL material attributed to the hydrolysis and UF treatment, which is greater than that previously published using similar hydrolysis and/or filtration technologies. Konrad, Kleinschmidt, and Lorenz (2013) obtained a PL concentrate following peptic hydrolysis and UF with a 2.5% increase in PL material. Morin et al. (2006) obtained a 3× enrichment of PL content following MF. In this study, it would appear that the lower temperatures used in the membrane process may have negatively influenced the transmission of the lipid components, which is in agreement with previous work (Astaire et al., 2003; Morin et al., 2006). Morin et al. (2007a) obtained a washed cream buttermilk sample with a 2.8 fold increase in PL material following MF and diafiltration. The values obtained for the more acidic PLs, PI and PS, are greater than that previously reported in the literature, 10.84 ± 0.04% PI and 9.54 ± 0.01% PS in the BMP and 15.25 ± 0.11% PI and 11.62 ± 0.03% PS in the 50 kDa R (Table 2). This increase is attributed to the sensitivity and precision of the CAD system and also the Folch lipid extraction method (Barry et al., 2016). It was noted that the % of SM was higher in the BMP compared with the 50 kDa R: 26.57 ± 0.04% and 20.97 ± 0.05%, respectively (Table 2). This decrease is attributed to the increase in PI thus leading to a lower calculated % of SM with respect to total PL.

HPLC-CAD chromatograms obtained for the BMP and 50 kDa R fractions (Fig. 5) demonstrate well defined peaks representing each of the five major PLs, namely phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC) and sphingomyelin (SM) and also glucosylceramide (GluCer). Sub-peaks of both PC (PC 1, PC 2), and SM (SM 1, SM 2) are evident in the chromatograms. These sub-peaks are due to the existence of different molecular species of PC and SM as already described (Barry et al., 2016; Deschamps, Chaminade, Ferrier, & Baillet, 2001; Fagan & Wijesundera 2004). From the chromatogram, the increase in PL material in the 50 kDa R (Fig. 5b) can be determined due to the higher concentration of PLs compared with that of the starting BMP (Fig. 5a): 6.16 ± 0.02% and 0.79± 0.00%, respectively. The total lipid and PL contents of 50 kDa P was 0.09 ± 0.02% and 0.00 ± 0.01%, respectively, thus confirming that there was no permeation or loss of PL during UF (Table 2). Secondly, protein digestion to 19% DH would appear to be essential to ensure sufficient permeation of smaller peptide

material through the UF 50 kDa membrane so that concentration of residual lipid components is maximised. Thus, the resulting retentate with PL concentration of 6.16 ± 0.02% total PL and non-detectable losses of PL was superior to that obtained using casein micelle disruption or coagulation-based separation support strategies used by others (Corredig et al., 2003; Roesch et al., 2004; Rombaut et al., 2007; Sachdeva & Buchheim, 1997). Thus, unlike previous researchers who utilised MF in their separations, this study proved that a combination of UF membrane and an optimised level of hydrolysed buttermilk protein prior to filtration enabled satisfactory PL enrichment to be accomplished.

4. Conclusion

The aim of the work presented here was to achieve an enrichment of phospholipids that exceeded previously reported values. This was carried out through the deployment of a twin strategy involving enzymatic hydrolysis to digest the milk proteins in a buttermilk substrate to facilitate subsequent UF permeation of the resulting peptide mixture.

Alcalase was determined as the most proteolytic enzyme for the protein hydrolysis with enzyme selection based on the greatest generation of smaller molecular mass peptides, 89.84% < 50 kDa. An optimum degree of hydrolysis of 19% with Alcalase ensured extensive hydrolysis of the milk proteins with almost complete digestion of the larger molecular mass material present in the starting BMP, 0.25 ± 0.02% and 92.10 ± 0.6% > 100 kDa, respectively, with pH adjustment identified as the preferred method of enzyme inactivation compared with noticeable aggregate formation, > 50 kDa, upon thermal treatment.

Finally, UF with a 50 kDa membrane was successful in reducing the protein content of the BMP hydrolysate from 31.40 ± 0.57% to 17.9 ± 0.19% in the retentate, while increasing lipid content of the latter from 6.84 ± 0.17% to 43.43 ± 0.61%, respectively. This 6-fold increase in lipid material was attributed to degradation, dissociation and subsequent permeation of the hydrolysed protein and concomitant increase in PL content of the 50 kDa retentate to 6.16 ± 0.02% total PL compared with 0.79 ± 0.00% total PL in the starting material.

This combined strategic approach has opened up new avenues for PL enrichment, highlighting the enhanced results obtained with the techniques outlined compared with results previously reported using similar techniques for separation of these techno- and bio- functionally attractive compounds.

Acknowledgements

The authors would like to acknowledge that the work herein was funded by the Food Institutional Research Measure (FIRM), project number: 10RD/TMFRC/709 administered by the Irish Department of Agriculture, Food & Marine. K. M. Barry was a Teagasc PhD Walsh Fellow supported by this FIRM project.

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Figure legends

Fig. 1. Size exclusion high performance liquid chromatography mass distribution profiles of buttermilk powder (dashed line) and hydrolysates generated during enzymatic screeing: A, Alcalase; B, Corolase PP; C, Neutrase; D, CPP + Alcalase; E, Neutrase + Alcalase.

Fig. 2. Size exclusion high performance liquid chromatography mass distribution profiles of buttermilk powder at different degrees of hydrolysis (% DH): A, 0% DH; B, 9% DH; C, 19% DH; D, 25% DH.

Fig. 3. Size exclusion high performance liquid chromatography mass distribution profiles of (A) unhydrolysed buttermilk powder, and 19% degree of hydrolysis buttermilk powder with (B) 20 min inactivation at 80 °C, or (C) inactivation with 0.1% TFA.

Fig. 4. Size exclusion high performance liquid chromatography mass distribution profiles of (A) 19% degrees of hydrolysis buttermilk powder (19% DH BMP) 50 kDa retentate and (B) 19% DH BMP 50 kDa permeate.

Fig. 5. High performance liquid chromatography-corona charged aerosol detector chromatogram of phospholipid composition of (A) buttermilk powder and (B) 50 kDa retentate of buttermilk powder hydrolysate.

Table 1

Molecular mass distributions of buttermilk powder hydrolysates during enzyme screening. [a]

Enzyme	Molecular mass distribution (%)					
	>100 kDa	100–75 kDa	75–50 kDa	50–30 kDa	30–10 kDa	< 10 kDa
None	92.10 ± 0.61	7.30 + 0.17	0.55 + 0.11	0.05 + 0.03		
Corolase PP (CPP)	32.43 ± 0.01	17.53 + 0.09	13.98 + 0.07	21.84 + 0.01	5.52 + 0.19	8.65 + 0.07
Neutrase	26.43 ± 0.00	5.56 + 0.32	4.05 + 0.12	29.11 + 0.07	15.56 + 0.51	19.28 + 0.03
Alcalase	0.25 ± 0.02	2.95+ 0.01	6.97 + 0.01	36.45 + 0.03	17.21+ 0.01	36.18 + 0.02
CPP + Alcalase	20.71 ± 0.05	6.71 + 0.09	4.31 + 0.07	34.31 + 0.10	16.92 + 0.11	17.03 + 0.02
Neutrase + Alcalase	17.29 + 0.09	4.91 + 0.06	4.63 + 0.07	34.25 + 0.12	18.33 + 0.10	20.59 + 0.01

[a] Distribution quantified with respect to calibration with known mass standards. Data are expressed as the average (+ % RSD) of three replicate hydrolysis reactions of the % of total area under the curve. Hydrolysis with Alcalase was selected as the way forward for producing BMP hydrolysate.

Table 2

Compositional analysis of buttermilk powder (BMP), 50 kDa retentate (50 kDa R) and 50 kDa permeate (50 kDa P) with respect to fat, protein, and total and individual phospholipids. [a]

Component	BMP	50 kDa R	50 kDa P
Fat (%)	6.84 ± 0.17	43.43 ± 0.61	0.09 ± 0.02
Protein (%)	31.40 ± 0.57	17.90 ± 0.19	39.31 ± 0.09
Total phospholipids (% sample)	0.79 ± 0.00	6.16 ± 0.02	0.00 ± 0.01
Individual phospholipids			
Phosphatidylinositol (PI)	10.84 ± 0.04	15.25 ± 0.11	
Phosphatidylethanolamine (PE)	21.66 ± 0.07	22.28 ± 0.04	
Glucosylceramide (GluCer)	0.07 ± 0.32	0.10 ± 1.14	
Phosphatidylserine (PS)	9.54 ± 0.01	11.62 ± 0.03	
Phosphatidylcholine (PC)	31.31 ± 0.01	29.78 ± 0.02	
Sphingomyelin (SM)	26.57 ± 0.04	20.97 ± 0.05	

[a] Fat and protein were determined using the Folch method (Barry et al., 2016) and the Kjeldahl method (ISO, 2014), respectively. Total phospholipid was determined by summation of each individual PL with respect to that extracted from the sample. Data for fat and total phospholipids are expressed as the average (± %RSD) of three repeats; those for protein are expressed as the average (± %RSD) of two repeats. Data for individual phospholipids are expressed as % of total PL, average (± %RSD) of duplicate injections of each sample in triplicate.

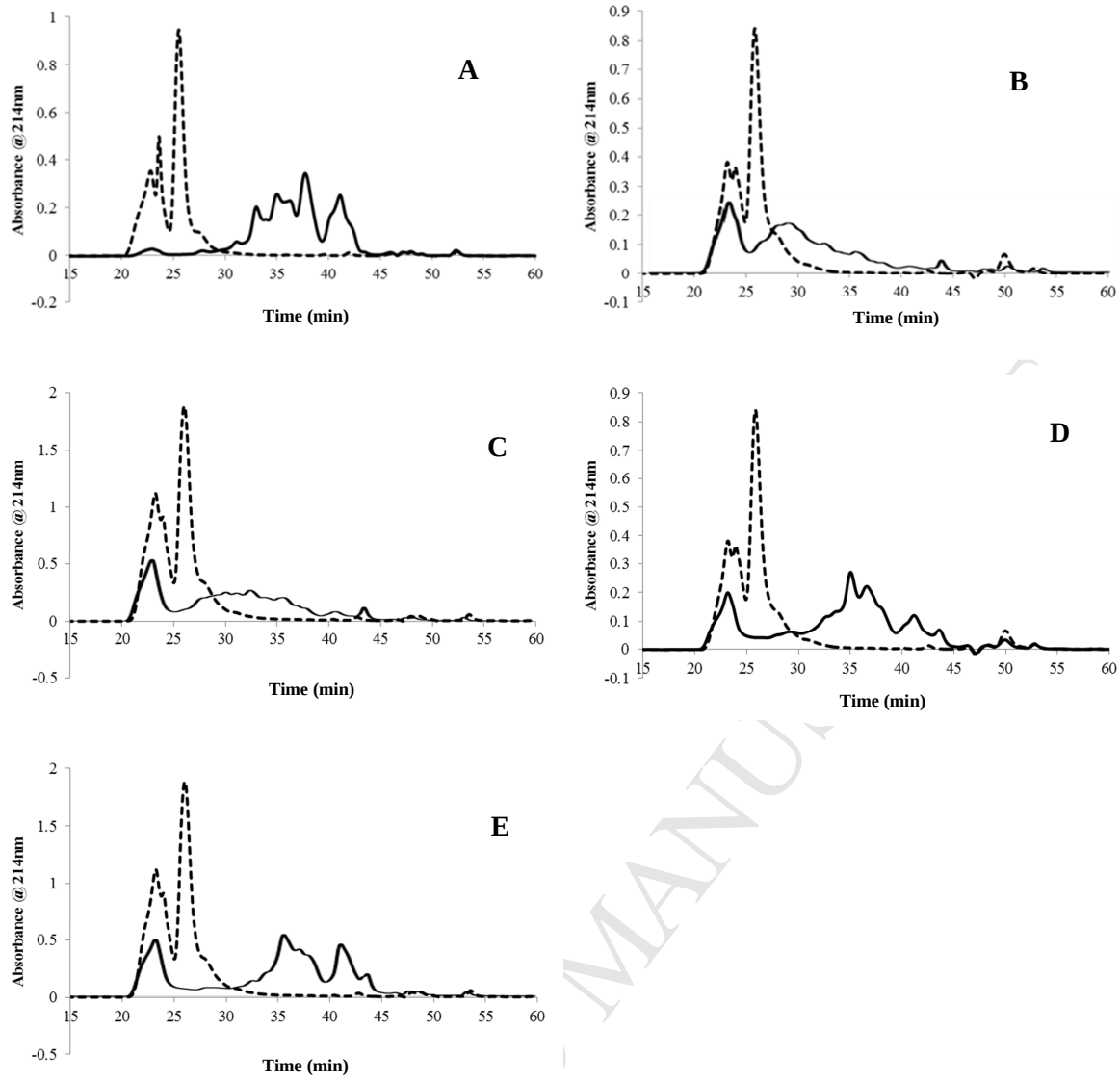

Figure 1

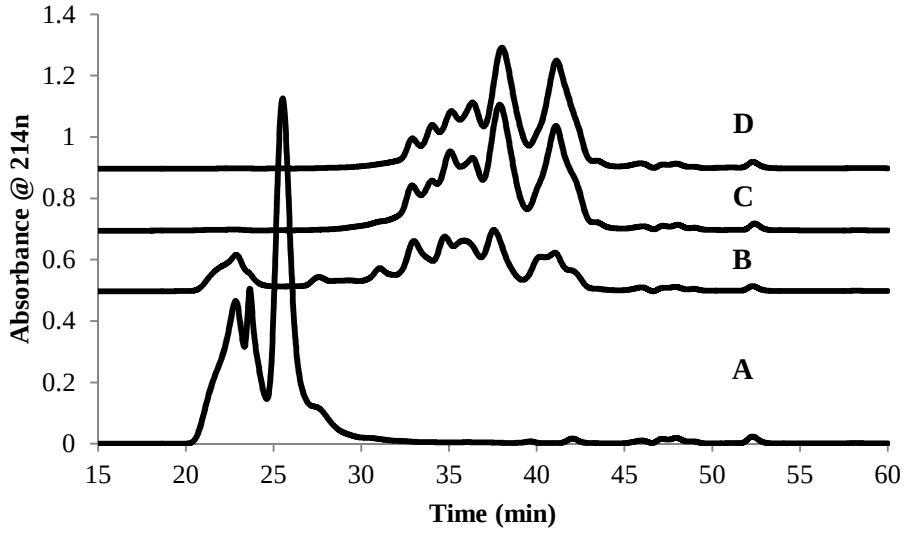

Figure 2

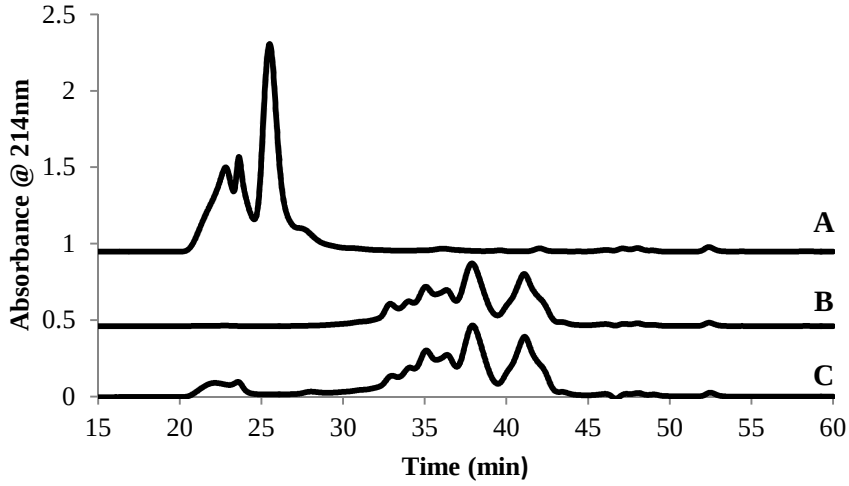

Figure 3

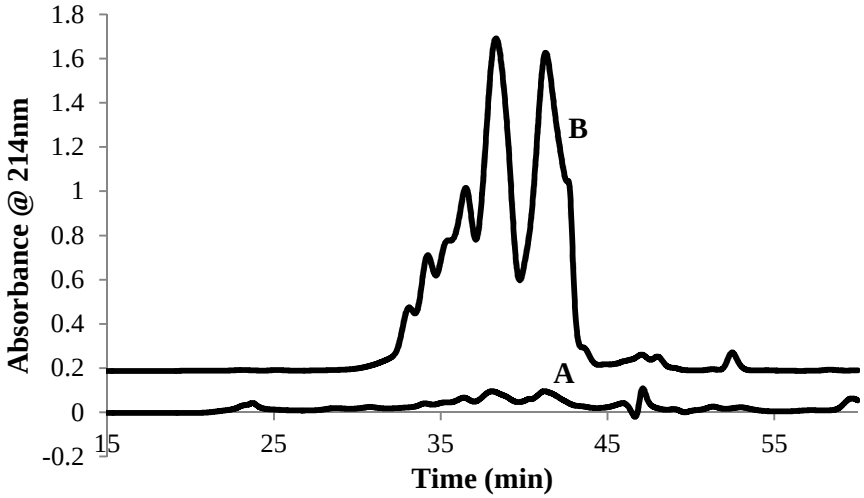

Figure 4

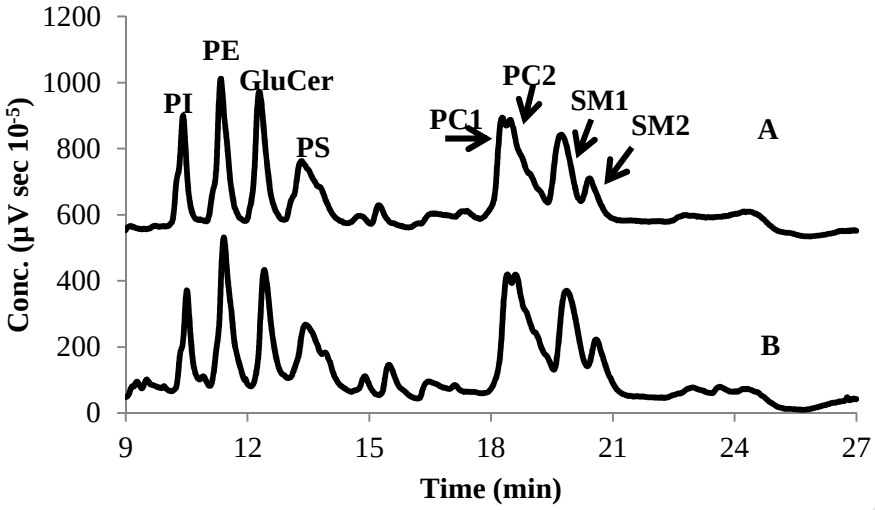

Figure 5